APPLICATION NOTE

Ionization and Fragmentation of Neutral and Acidic Glycosphingolipids with a Q-TOF Mass Spectrometer Fitted with a MALDI Ion Source

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This paper reports the use of a quadrupole time-of-flight (Q-TOF) mass spectrometer fitted with a matrix-assisted laser desorption/ionization (MALDI) ion source for the analysis of neutral and acidic glycosphingolipids. All compounds gave strong $[M + Na]^+$ ions with 2,5-dihydroxybenzoic acid as the matrix, with no loss of sensitivity with increasing mass as was observed from the corresponding ions produced by electrospray. Neutral glycosphingolipids showed negligible in-source fragmentation but sialylated compounds fragmented by loss of sialic acid. However, these losses were not accompanied by unfocused post-source-decay ions as observed with MALDI-reflectron-TOF instruments. The MS/MS spectra were almost identical to those obtained by electrospray. Fragmentation of all compounds was mainly by glycosidic cleavage to give ions, both with and without the ceramide moiety, which defined the carbohydrate chain sequence. Weak ions which defined the sphingosine chain length and abundant ions, produced by loss of the acyl chain, were present when this chain contained a 2-hydroxy group. The technique was applied to the identification of ceramide–trihexosides present in tissues from mice genetically modified to model one of the glycolipid storage diseases (Fabry disease). (J Am Soc Mass Spectrom 2001, 12, 1220–1225) © 2001 American Society for Mass Spectrometry

Glycosphingolipids (GSLs) consist of a non-polar lipid (ceramide) portion that acts as a membrane anchor and a water-soluble carbohydrate moiety, and are ubiquitous components of eukaryotic plasma membranes [1]. They have many cellular functions [2] such as cell–cell recognition, cell growth and signal transduction. Structural analyses have been carried out by mass spectrometry for many years and have been the subject of several reviews (see, for example [3–8]. Early work was performed using fast-atom bombardment (FAB) ionization but more recently electrospray and MALDI ionization techniques have been successfully used.

Analysis of GSLs by FAB mass spectrometry requires a relatively large quantity of sample [5, 9]. By comparison, MALDI mass spectrometry can produce signals from much smaller amounts of material [10, 11] without the need for derivatization. The resulting spectra contain few, if any, fragment ions and thus, fragmentation studies require additional stages of analysis. Fragmentation from samples ionized by MALDI and recorded with TOF mass analyzers is generally restricted to that obtained by post-source decay (PSD) [10, 12], a technique that produces fragment ions with relatively poor resolution. However, instruments are now available in which MALDI has been interfaced with a tandem quadrupole-TOF analyzer [13] and which have been shown to provide excellent MS/MS data from proteins [13–15] and carbohydrates [16]. This paper reports the use of this type of instrument for the analysis of glycosphingolipids.

Experimental

Materials

GSLs, all from bovine brain, except glucosylceramide which was from human Gaucher's spleen, were obtained from Sigma, (Poole, Dorset, UK) and used as supplied. All solvents were of analytical grade and were purchased from Sigma; the MALDI matrices, 2,5-dihydroxybenzoic acid (DHB), 1,4-dihydroxynaphthoic acid, 1,3,5-trihydroxyacetophenone (THAP) and 2-(4'-hydroxyphenyl)azobenzoic acid (HABA) were

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from Aldrich Chemical Co. Ltd., (Poole, Dorset, UK). Dextran sugars were obtained from Fluka Biochemika (Poole, UK). GSLs from the liver of Fabry mice were purified as described below. Silica 60 was purchased from Merck, (Darmstadt, Germany) and conditioned overnight at 80 °C before use.

Extraction of Glycosphingolipids from Fabry Mouse Liver and Kidney

GSLs were extracted with a mixture of chloroform: methanol (2:1, vol:vol, 2 ml) from 50 mg of tissue [17] dispersed into 1 ml of water by shaking for 3 h. The mixture was centrifuged at 3000 rpm for 10 min, the supernatant was removed, and the process was repeated. Pooled supernatants were freeze-dried overnight and purified by silicic acid chromatography. Neutral GSLs were eluted with acetone:methanol (9:1 vol:vol, 9 ml) [18] and dried (nitrogen stream).

MALDI Mass Spectrometry

A mixture of sample (1 μl, 100 pmol) and matrix (1 μl of a saturated solution of 2,5-DHB in acetonitrile) were crystallized on the MALDI target. Positive ion reflectron MALDI and PSD spectra were obtained with a Micromass TOFSpec 2E mass spectrometer equipped with delayed extraction (Micromass UK, Ltd., Wythenshawe, Manchester). The instrument was operated as follows: accelerating voltage, 20 kV; pulse voltage, 3200 V; laser repetition rate of 10 Hz and calibrated externally with hydrolyzed dextran sugars.

Electrospray Mass Spectrometry

Nanoflow electrospray mass spectra were obtained with a Micromass Q-TOF mass spectrometer (Z-spray ion source). Samples (10 μl, 100 pmol), in chloroform: methanol (2:1, vol/vol), were infused at 400 nL/min. Operating conditions were: needle voltage, 3kV; ion source temperature, 100 °C; collision gas, argon at 20 psi, spectrum acquisition time, 1 s.

MALDI-Q-TOF Mass Spectrometry

The mass spectrometer was a Micromass Q-TOF instrument fitted with an experimental MALDI ion source as described earlier [16]. Air was introduced to give a pressure of 0.1 mbar to provide collisional cooling of the ion beam to thermal levels in the hexapole ion guide [13]. Argon was used as the collision gas and the voltage on the collision cell was varied by about $\pm$10 V around the mean value (usually about 85 V). The mass window for precursor ion selection was about four mass units.

Results and Discussion

Neutral Glycosphingolipids

Figure 1 shows a comparison of the MS spectra of a mixture of neutral GSLs (approximately 100 pmoles of each was sampled, of which about 10% was used) recorded by MALDI using DHB as a matrix on the MALDI-Q-TOF instrument (Figure 1a), by electrospray (Figure 1b), and with a conventional MALDI-TOF instrument (Figure 1c). The MALDI-Q-TOF spectrum (Figure 1a) was similar to the MALDI spectrum recorded directly with the reflectron-TOF instrument (Figure 1c); neither spectrum showed the loss of sensitivity with increasing mass that was observed with the electrospray instrument (Figure 1b), an observation similar to that made earlier with N-linked carbohydrates [16]. No significant in-source fragmentation occurred during MALDI ionization. However, the higher laser power used by the MALDI Q-TOF instrument produced a slightly higher background than in the reflectron-TOF spectrum.

Acidic (Sialo-) Glycosphingolipids

Unlike spectra of neutral GSLs, the spectrum of the sialylated ganglioside GM1 recorded with the MALDI-TOF instrument (data not shown) showed extensive fragmentation by loss of sialic acid [10, 11, 19] leading to both focused (*m/z* 1277.7 and 1305.6) in-source fragments and unfocused, post-source or metastable ions (*m/z* 1317.2 and 1345.2) [20]. The positive ion MALDI-Q-TOF spectrum of this compound, on the other hand, although showing significant in-source fragmentation contained no metastable ions. Less fragmentation was observed with neutral matrices, such as THAP, or by using electrospray ionization, the latter observation paralleling those made by Penn et al. [21].

Additional fragmentation of acidic glycolipids was observed with the MALDI-Q-TOF instrument to give the ions at *m/z* 940.7 and 912.7 (20% relative abundance) by loss of Galβ1 $\rightarrow$ 3GalNAc from *m/z* 1305.9 and 1277.9 respectively ($Y_{2\beta}$ cleavage) (Domon and Costello [22] nomenclature), and ions at *m/z* 1552.9 and 1524.9 (20%) by loss of carbon dioxide [10]. Two weak (10%) ions at *m/z* 1441.9 and 1413.8. appeared to be adducts with a dehydration product (136 mass units) of the matrix as they were absent from the spectra recorded from 1,4-dihydroxynaphthoic acid (an analogue of DHB), HABA or THAP, and from the corresponding MS/MS spectra.

MS/MS Spectra of Neutral Glycosphingolipids

The MS/MS spectra of neutral GSLs, such as those from GA_2 obtained on the MALDI-Q-TOF instrument, (Figure 2a) were almost identical to those obtained on the electrospray Q-TOF instrument (Figure 2b) demonstrating effective decoupling of the ion source from the analytical stage of the instrument. The MALDI-PSD

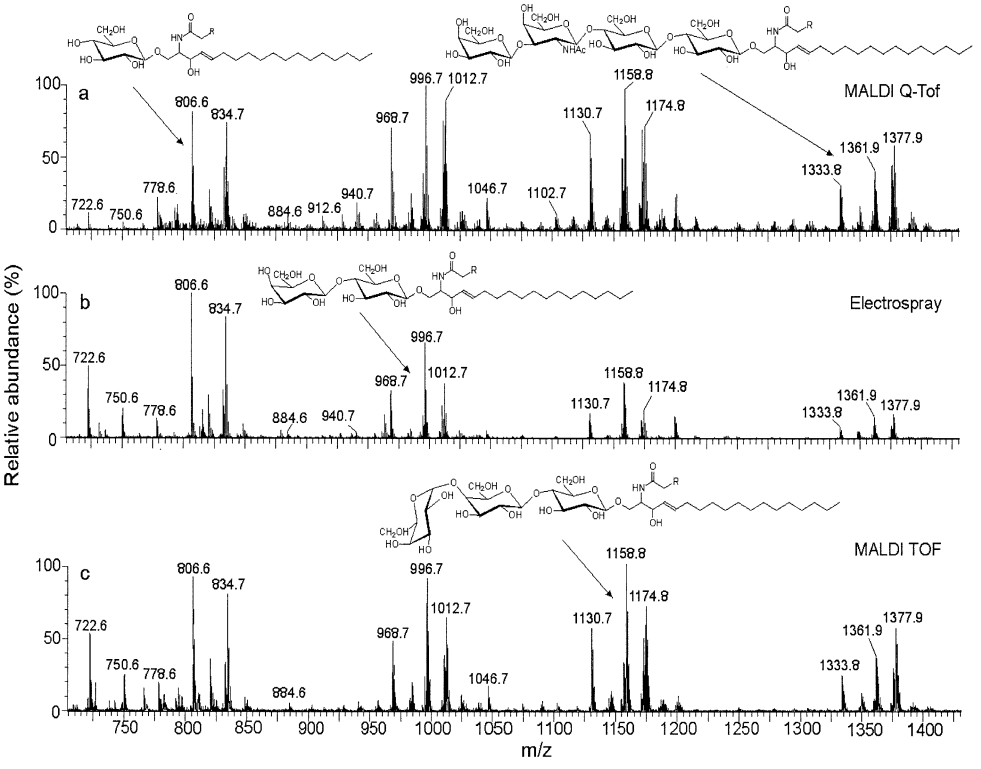

Figure 1. Mass spectra of a mixture of neutral glycosphingolipids recorded (**a**) by MALDI with the MALDI Q-TOF mass spectrometer, (**b**) by electrospray with the Q-TOF mass spectrometer, and (**c**) by MALDI with a reflectron-TOF mass spectrometer. R is used to represent heterogeneity in the acyl chain. Acyl groups contain from 16 to 24 carbon atoms.

spectrum recorded with a reflectron-TOF instrument, on the other hand (Figure 2c), although showing the same series of Y ions, as reported by others [19], was less informative and the fragment ions were poorly focused.

The major ions seen in both MALDI and electrospray MS/MS spectra were due to Y-type glycosidic cleavages of the glycan chain (*m/z* 912.7, 750.6, 588.5 in Figure 2) involving loss of a hydroxylic hydrogen atom, as shown by deuterium labeling [23]. This is in line with earlier studies on the cleavages of complex sugars [24]. Other fragment ions are annotated in Figure 2. The prominent ions formed by loss of the entire carbohydrate moiety together with H_2CO reported by Hsu and Turk [23] in the spectra of lithium adducted glycolipids, and which we have also found in electrospray spectra, were absent. Lithium has previously been reported to catalyze more fragmentation from carbohydrates than sodium [25].

The ion at *m/z* 264, derived from the sphingosine chain [23, 26–29] and defining its length as C-18, was weak but quite distinct in the MALDI Q-TOF spectrum as shown by the inset to Figure 2. Its relative abundance fell as the number of sugar residues increased but was generally more abundant in these MALDI spectra than in the ones recorded with the electrospray Q-TOF instrument. The ion is interesting in that it is a protonated species derived from a sodiated molecular ion. Several structures have been proposed for this ion; for example, Olling et al. [28] favor a structure containing a carbonium ion whereas Hsu and Turk [23] have proposed a cyclic ethyleneiminium ion.

GSLs containing 2-hydroxy fatty acyl groups, such as that shown for the LacCer in Figure 3 (2-hydroxy-24:0 acyl), were characterized by an abundant ion produced by loss of the hydroxy-acyl group (*m/z* 646.4) [23, 28–30]. A further loss of galactose was observed from this ion to give *m/z* 484.3 and a weaker ion at *m/z* 674.3 was formed by cleavage between the acyl carbonyl group and the C_2-carbon atom. In other respects, the fragmentation was similar to that of GSLs containing unsubstituted fatty acids.

MS/MS Spectra of Acidic Glycosphingolipids

MS/MS spectra of acidic GSLs were similar to those of the neutral compounds with the major fragments being due to loss of the sialic acid moieties. Where the sialic acids formed sodium salts, loss of sialic acid loss was suppressed, presumably because of the absence of the labile acidic proton that was presumed to migrate during the Y cleavage. Where sialic acids were chained, as in the ganglioside GD3 (Neu5Ac2 → 8 Neu5Ac2 → 3Gal1 → 4Glc1 → Cer), the presence of the Neu5Acα2 → 8Neu5Ac group in this molecule was reflected by the B ions at *m/z* 314.1 and 605.2 corresponding to the sodium adducts of fragments containing one and two sialic acids, respectively. Where a single sialic

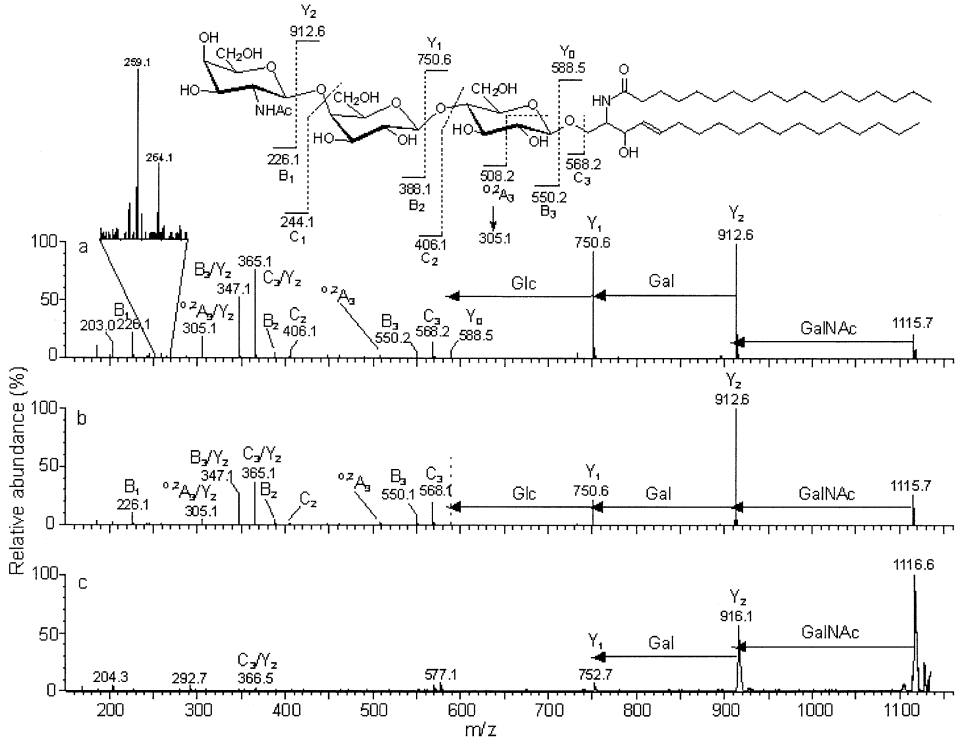

Figure 2. MALDI MS/MS spectra of the neutral glycosphingolipid GA_2 (C_{18} sphingosine and acyl groups) recorded with (**a**) the MALDI Q-TOF mass spectrometer and (**b**) the reflectron-TOF mass spectrometer. (**c**) Shows the corresponding PSD spectrum. The inset to spectrum (**a**) shows a magnification of the region containing the ion at *m/z* 264.1.

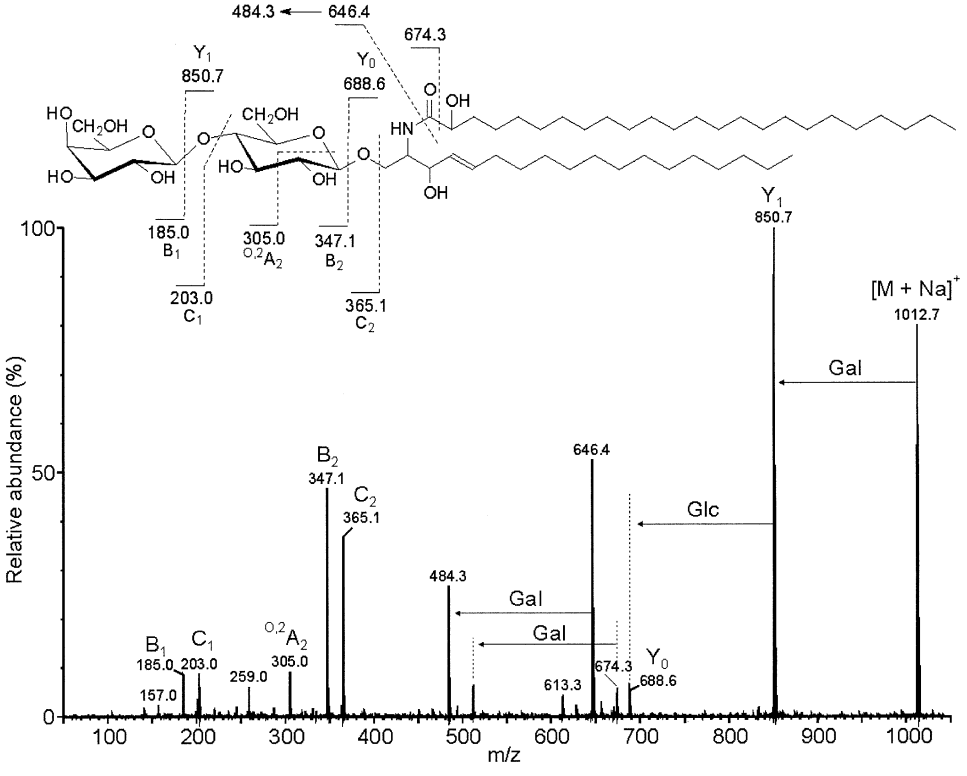

Figure 3. MALDI MS/MS spectrum of lactosylceramide containing a 2-hydroxy fatty acyl chain recorded with the MALDI Q-TOF mass spectrometer.

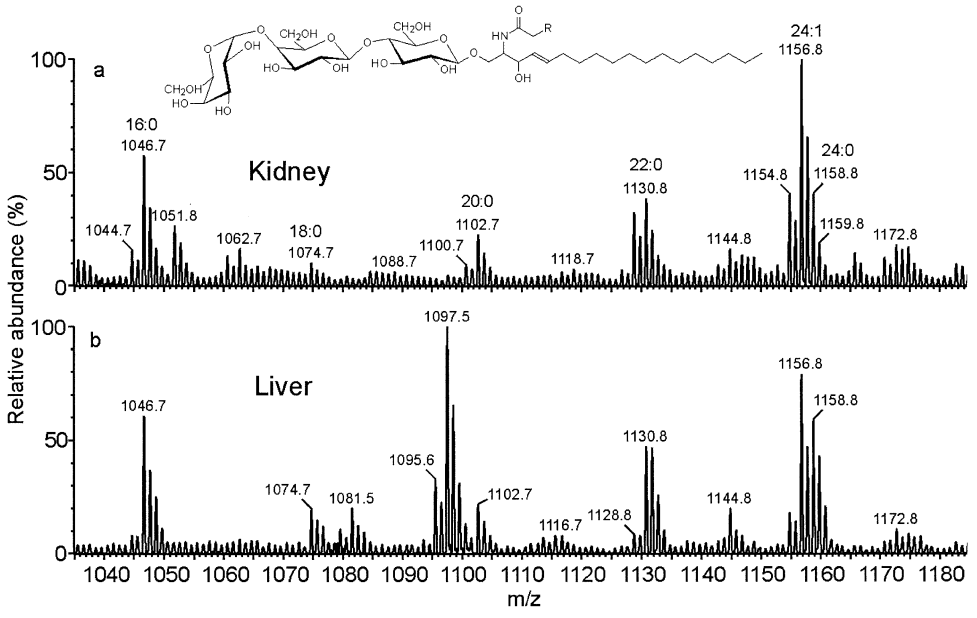

Figure 4. MALDI Mass spectra of trihexosylceramides from Fabry mouse kidney (**a**) and liver (**b**). Peaks are labeled with the acyl chain lengths.

acid was substituted on galactose, as in the spectrum of GD_{1a}, only the mono-sialyl ion at *m/z* 314.1 was present (data not shown).

Application to Glycosphingolipids from Mouse Models of Glycolipid Storage Diseases

The GSL lysosomal storage diseases [31] are a group of relatively rare genetic disorders in which the genes encoding the catabolic enzymes for GSLs are mutated, leading to accumulations of substrate. Deficiency of α-galactosidase A leads to accumulation of ceramide trihexosides (globotriaosylceramide) in the liver, heart, spleen, kidney, vascular endothelial cells, and plasma to give a condition known as Fabry disease [32]. Figure 4 shows the MALDI Q-TOF spectra of ceramide trihexoside from the kidney (Figure 4a) and liver (Figure 4b) of a mouse genetically altered to reproduce Fabry disease [17]. The spectra showed a similar range of glycolipids in both tissues (the abundant ion at *m/z* 1097.6 in the sample from the liver (Figure 4b) was not a glycolipid as shown by its MS/MS spectrum). The MS/MS spectra confirmed the presence and sequence of the trihexose moiety. An ion at *m/z* 264 in the spectra of all the major compounds, confirmed the sphingosine as being d18:1 and the heterogeneity as being in the acyl group. Other studies on the ceramide portion of murine glycolipids have shown similar profiles [33] indicating that the Fabry disease has not significantly affected the ceramide portion of the molecule.

Conclusions

Collisional cooling of the ion beam enabled both MS and MS/MS spectra to be recorded with the Q-TOF instrument from the MALDI ion source. GSLs produced singly charged $[M + Na]^+$ ions which reflected the relative amounts of substance present in the sample and gave spectra that were very similar to those recorded with a reflectron-TOF instrument. Consequently, MS/MS spectra could be recorded with the Q-TOF instrument from the $[M + Na]^+$ ions from all components in mixtures. The more common electrospray ion source failed to produce $[M + Na]^+$ ions from the larger compounds. The effective decoupling of the ion production from the ion analysis stages enabled MS/MS spectra to be obtained that were independent of the method of ion production and provided a means of obtaining high quality fragmentation spectra from underivatized GSLs.

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